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Antiproliferative Effects of 1α,25-Dihydroxyvitamin D_3 and Vitamin D Analogs on Tumor-Derived Endothelial Cells

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Although there is abundant evidence that 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] inhibits the growth of several cancer cell types, inhibition of angiogenesis may also play a role in mediating the antitumor effects of 1,25-$(OH)_2D_3$. We examined the ability of 1,25-$(OH)_2D_3$ to inhibit the growth of tumor-derived endothelial cells (TDECs) and normal endothelial cells and to modulate angiogenic signaling. 1,25-$(OH)_2D_3$ inhibited the growth of TDECs from two tumor models at nanomolar concentrations, but was less potent against normal aortic or yolk sac endothelial cells. The vitamin D analogs Ro-25–6760, EB1089, and ILX23–7553 were also potent inhibitors of TDEC proliferation. Furthermore, the combination of 1,25-$(OH)_2D_3$ and dexamethasone had greater activity than either agent alone. 1,25-$(OH)_2D_3$ increased vitamin D receptor and $p27^{Kip1}$ protein levels in TDECs, whereas phospho-ERK1/2 and phospho-Akt levels were reduced. These changes were not observed in normal aortic endothelial cells. In squamous cell carcinoma and radiation-induced fibrosarcoma-1 cells, 1,25-$(OH)_2D_3$ treatment caused a reduction in the angiogenic signaling molecule, angiopoietin-2. In conclusion, 1,25-$(OH)_2D_3$ and its analogs directly inhibit TDEC proliferation at concentrations comparable to those required to inhibit tumor cells. Further, 1,25-$(OH)_2D_3$ modulates cell cycle and survival signaling in TDECs and affects angiogenic signaling in cancer cells. Thus, our work supports the hypothesis that angiogenesis inhibition plays a role in the antitumor effects of 1,25-$(OH)_2D_3$. (*Endocrinology* 143: 2508–2514, 2002)

THE ACTIVE FORM of vitamin D, 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] is a key regulator of calcium homeostasis (1) and has antitumor activity in a wide variety of tumor types (2). Low nanomolar concentrations of 1,25-$(OH)_2D_3$ can directly inhibit the proliferation of several types of cancer cells (2, 3). Interestingly, we have observed that 1,25-$(OH)_2D_3$ also inhibits the growth of tumors generated by a prostate cancer cell line that is relatively resistant to 1,25-$(OH)_2D_3$ *in vitro* (Yu, W.-D., and C. S. Johnson, unpublished observations). A possible explanation for these results is that 1,25-$(OH)_2D_3$ acts by inhibiting angiogenesis in these tumors. Angiogenesis is known to be required for growth of tumors larger than a few millimeters (4), and agents that selectively inhibit endothelial cells have been shown to limit tumor growth (5, 6). Further, endothelial cells are attractive therapeutic targets because they have greater genomic stability than cancer cells and are less likely to rapidly develop resistance (7).

Previous reports support the hypothesis that 1,25-$(OH)_2D_3$ inhibits tumor angiogenesis. Expression of the vitamin D receptor (VDR) has been observed in endothelial cells (8), and 1,25-$(OH)_2D_3$ and its analogs inhibit embryonic angiogenesis in chick chorioallantoic membranes (9). Decreased vessel density and VEGF expression have also been observed in various tumor models after treatment with 1,25-$(OH)_2D_3$ (10, 11). An inhibitory effect for 1,25-$(OH)_2D_3$ against VEGF-stimulated growth of bovine aortic endothelial cells has also been observed, as well as inhibition of sprouting and elongation (12). However, the responsiveness of endothelial cells that originate from tumors has not been examined directly.

We and others have previously isolated tumor-derived endothelial cells (TDECs) and shown that they differ in several ways from endothelial cells derived from normal tissues (13–16); therefore, all endothelial cells may not respond the same to 1,25-$(OH)_2D_3$. Here, we examined the antiproliferative effects of 1,25-$(OH)_2D_3$ in TDECs and endothelial cells derived from normal tissues.

As use of 1,25-$(OH)_2D_3$ as a single agent is limited by its hypercalcemic effects (17–19), we also investigated the effects of three vitamin D analogs, which are less hypercalcemic, on TDECs. In addition, we tested the ability of the combination of 1,25-$(OH)_2D_3$ and dexamethasone (DEX) to inhibit the proliferation of TDECs, as we have shown that DEX enhances the antitumor activity and reduces the toxicity of 1,25-$(OH)_2D_3$ (20). Further, we compared the expression of VDR and effects on growth and survival signaling pathways in TDECs and endothelial cells derived from normal tissues. Finally, we examined the possibility that 1,25-$(OH)_2D_3$ affects angiogenic signaling between cancer cells and endothelial cells.

Abbreviations: Ang-2, Angiopoietin-2; DEX, dexamethasone; 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; MAE, murine aortic endothelial cells; MYSEC, murine yolk sac endothelial cells; PARP, poly(ADP-ribose) polymerase; Rb, retinoblastoma; RIF, radiation-induced fibrosarcoma; SCC, squamous cell carcinoma; TDECs, tumor-derived endothelial cells; VDR, vitamin D receptor.

Materials and Methods

Chemicals and reagents

1,25-$(OH)_2D_3$ and the vitamin D analogs 7553 (1,25-dihydroxy-16-ene-23-yne-cholecalciferol, ILX23–7553, Ilex) and 6760 (1,25-dihydroxy-16-ene-23-yne-26,27-hexafluoro-19-nor-cholecalciferol, Ro-25-6760,

Hoffmann-La Roche, Inc., Nutley, NJ) were provided by Dr. Milan R. Uskokovic (Hoffmann-LaRoche, Inc.). EB1089 [1(*S*),3(*R*)-dihydroxy-20(*R*)-(5′-ethyl-5′-hydroxy-hepta-1′(*E*),3′(*E*)-dien-1′-yl)-9,10-secopregna-5(Z),7(*E*),10(19)-triene, Leo Pharmaceuticals, Ballerup, Denmark) was a gift from Dr. Lise Binderup (Leo Pharmaceuticals). 1,25-$(OH)_2D_3$, 6760, and 7553 were reconstituted in ethanol at 1 mg/ml. EB1089 was dissolved in isopropanol to make a 4-mM stock solution. DEX 21-phosphate (Sigma, St. Louis, MO) was dissolved in dH_20 to 25 mg/ml. All agents were diluted to the appropriate final concentrations in tissue culture medium immediately before use.

Cells and model systems

The squamous cell carcinoma (SCC) VII/SF murine SCC cells, which were derived from a spontaneously arising tumor of the C3H mouse (21), and radiation-induced fibrosarcoma-1 (RIF-1) cells, which were derived from a RIF in the C3H mouse (22), were maintained in RPMI 1640 supplemented with 14% FBS and 1% penicillin-streptomycin sulfate.

We previously reported the isolation and characterization of TDECs from both the RIF-1 (13) and SCC VII/SF (15) tumor models. Briefly, tumors were harvested, and a single cell suspension was prepared using an enzyme cocktail including collagenases and ribonucleases. Cells were stained with antibodies specific for the endothelial cell markers, angiotensin-converting enzyme for RIF-1 and platelet-endothelial cell adhesion molecule for SCC, followed by a fluorescein isothiocyanate-linked secondary antibody. Cells were then sorted by fluorescence-activated flow cytometry based on the level of fluorescein isothiocyanate staining. Murine yolk sac endothelial cells (MYSEC) and murine aortic endothelial cells (MAE) cells were a gift from Dr. Robert Auerbach (University of Wisconsin, Madison, WI) and were derived using a similar method. For all experiments, the endothelial cells were grown in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin sulfate on 1% gelatin-conditioned plastic.

Crystal violet assay

As described previously (3), cells were plated in 96-well plates and allowed to attach for 24 h before treatments were added. Cells were then incubated for 72 h before harvesting by staining with crystal violet, and the OD was read at 540 nM. The percent inhibition was calculated using the following equation: $(1 - [(OD_{treated} - OD_{background})/(OD_{control} - OD_{background})]) \times 100$ = % inhibition.

In vitro treatments, preparation of whole cell lysates, and Western blot analysis

Sample preparation and Western blot analysis were performed essentially as described previously (23). Antibodies used include polyclonal rabbit anti-VDR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), polyclonal rabbit antimouse p21 (BD Pharmingen, San Diego, CA), polyclonal rabbit antimouse $p27^{Kip1}$ (BD Pharmingen), mouse monoclonal anti-phospho-ERK (Santa Cruz Biotechnology, Inc.), polyclonal rabbit antiphospho-Akt (Ser^{473}; Cell Signaling Technology, Beverly, MA), purified mouse antihuman Rb (BD Pharmingen), mouse monoclonal anti-p53 (Santa Cruz Biotechnology, Inc.), polyclonal rabbit anti-Fas (Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-poly(ADP-ribose) polymerase (anti-PARP; Enzyme Systems Products, Livermore, CA), and purified rabbit antimouse angiopoietin-2 (APC-2; Calbiochem, San Diego, CA). Antirabbit and antimouse horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Arlington Heights, IL) were used.

Results

1,25-$(OH)_2D_3$ inhibits the proliferation of tumor-derived endothelial cells

We used a crystal violet assay to test the antiproliferative effects of 1,25-$(OH)_2D_3$ treatment on TDECs derived from a murine squamous cell carcinoma ($TDEC_{SCC}$) and a murine radiation-induced fibrosarcoma ($TDEC_{RIF}$), as well as normal endothelial cells derived from yolk sac (MYSEC) and aortic (MAE) tissues (Fig. 1). Considerable variation was observed in responsiveness to the antiproliferative effects of 1,25-$(OH)_2D_3$ among these endothelial cell types. As the doubling times for all four cell types were between 25–28 h, the large differences in sensitivity to 1,25-$(OH)_2D_3$ that were observed cannot be accounted for by variations in growth rate. Interestingly, the two TDEC types were more sensitive than the endothelial cells from either normal tissue. In addition, the potency of 1,25-$(OH)_2D_3$ on both $TDEC_{SCC}$ and $TDEC_{RIF}$ (IC_{50} = 2.6 and 10.9 nM, respectively) was comparable to what was observed for the corresponding tumor cells (3).

Vitamin D analogs and combination treatments also inhibit TDEC proliferation

Since the hypercalcemia induced by 1,25-$(OH)_2D_3$ limits the dose that can be given safely (17–19), other vitamin D-based therapies have also been considered, including use of vitamin D analogs with less propensity to increase serum calcium levels (24) and combinations of 1,25-$(OH)_2D_3$ with agents, such as DEX, that reduce the calcemic effects (20). Here we demonstrate that improved antiproliferative effects against TDECs can be achieved using the same drug concentration by either replacing 1,25-$(OH)_2D_3$ with a vitamin D analog or combining 1,25-$(OH)_2D_3$ with DEX. The analogs EB1089 (IC_{50} = 0.44 nM) and 7553 (IC_{50} = 0.39 nM) were more potent than 1,25-$(OH)_2D_3$, whereas 6760 (IC_{50} = 2.7 nM) was similar to 1,25-$(OH)_2D_3$ in potency (Fig. 2A). The combination of 1,25-$(OH)_2D_3$ and DEX also had greater antiproliferative effects than either agent alone on $TDEC_{SCC}$ (Fig. 2B). These results do not take into account the additional advantage that these strategies may allow higher doses to be given without dose-limiting hypercalcemia.

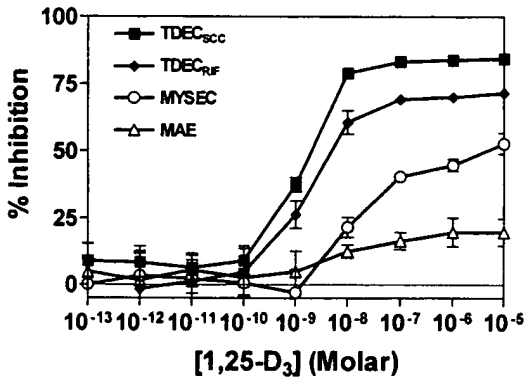

FIG. 1. 1,25-$(OH)_2D_3$ inhibits the growth of tumor-derived endothelial cells *in vitro*. The antiproliferative effects of 1,25-$(OH)_2D_3$ were examined in four different endothelial cell types: $TDEC_{SCC}$ (■), $TDEC_{RIF}$ (♦), MYSEC (○), and MAE (△). Cells were treated for 72 h with varying concentrations of 1,25-$(OH)_2D_3$, and the percent inhibition of growth compared with untreated controls was determined using a crystal violet assay. Each point represents the mean of four wells ± SD. The results are representative of at least two independent experiments. The IC_{50} values for 1,25-$(OH)_2D_3$ in SCC and RIF-1 tumor cells were previously determined to be 4.2 and 33.3 nM, respectively.

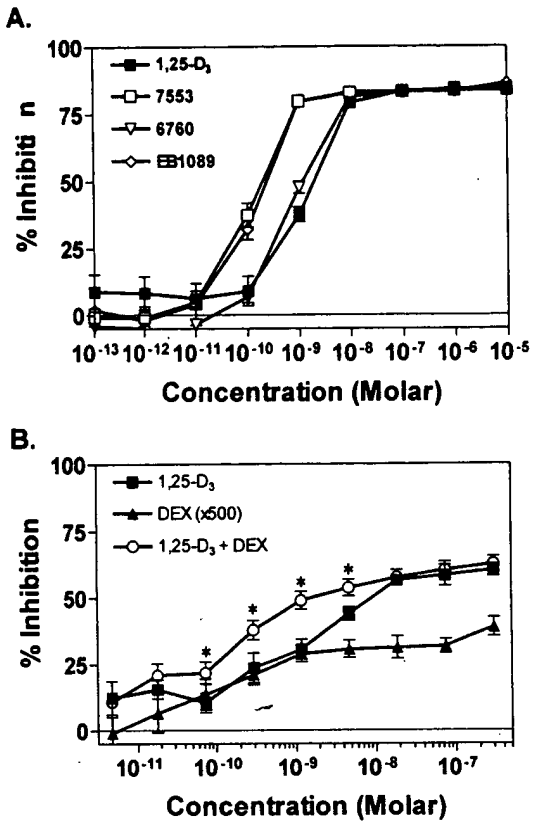

FIG. 2. Antiproliferative effects of vitamin D analogs and the combination of 1,25-$(OH)_2D_3$ on $TDEC_{SCC}$. A, Crystal violet assays were performed to assess the antiproliferative effects of three vitamin D analogs compared with 1,25-$(OH)_2D_3$ in $TDEC_{SCC}$. Cells were treated for 72 h with a wide range of doses of 7553 (□), 6760 (▽), EB1089 (◇), or 1,25-$(OH)_2D_3$ (■). Percent inhibition relative to untreated cells was then determined. The IC_{50} for 7553 was previously determined to be 0.3 and 59.5 nM for SCC and RIF-1 tumor cells, respectively. B, Crystal violet assays were performed to assess the antiproliferative effects of the combination of 1,25-$(OH)_2D_3$ and DEX on $TDEC_{SCC}$. Cells were treated with a range of concentrations of DEX (72 h; ▲), 1,25-$(OH)_2D_3$ (48 h; ■), or both (○). A constant ratio of 1,25-$(OH)_2D_3$ to DEX was used (1:500). Percent inhibition relative to untreated cells was then determined. Each *point* represents the mean of four wells ± SD. *Asterisks* indicate values for the combination that were significantly different from the value with either agent alone ($P < 0.05$, by *t* test). The results are representative of at least two independent experiments.

Expression of VDR varies among endothelial cells of different origins

As we observed variations in the responsiveness to 1,25-$(OH)_2D_3$ among endothelial cell types derived from different tissues, we examined whether the sensitivity to 1,25-$(OH)_2D_3$ could be explained by differences in the expression of the VDR. We performed Western blots for VDR in four endothelial cell types after treatment with 1,25-$(OH)_2D_3$ and/or DEX (Fig. 3). Although the low levels of VDR found in MAE correlated with the relative resistance to 1,25-$(OH)_2D_3$, MYSEC expressed similar levels of VDR to TDECs after treatment with 1,25-$(OH)_2D_3$, but were significantly less sensitive, suggesting that other factors are involved. Furthermore, addition of DEX caused a further induction of VDR in $TDEC_{SCC}$ and $TDEC_{RIF}$; similar results have been observed in SCC cells (25).

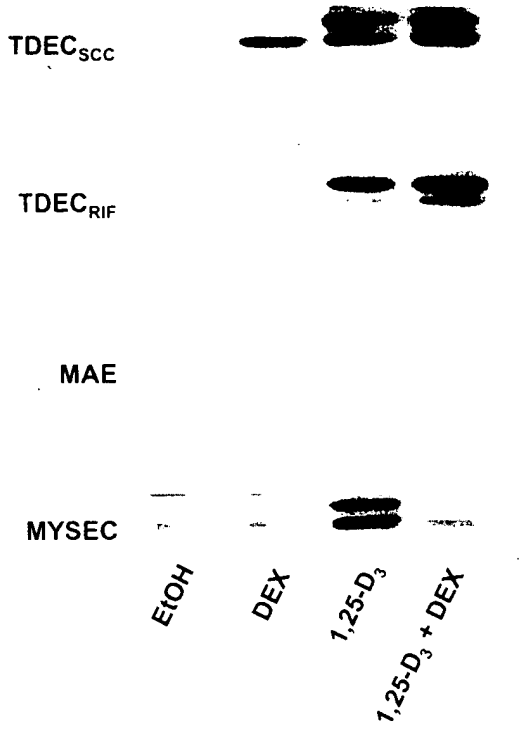

FIG. 3. Expression of VDR in endothelial cells of different origins. Whole cell lysates of four different endothelial cell types were analyzed for the expression of VDR. Lysates were prepared from all cell types after treatment with the ethanol vehicle control, 1,25-$(OH)_2D_3$ (10 nM) with or without DEX (500 nM), or DEX alone for 48 h. Western blots were then performed loading equal amounts of protein for all cell types and treatment groups and using an antibody specific for VDR. The cell types used include $TDEC_{SCC}$, $TDEC_{RIF}$, MAE, and MYSEC. Very low levels of VDR were detectable in MAE on a long exposure. Similar results were observed in two independent experiments.

Effects of 1,25-$(OH)_2D_3$ on key growth/survival signals and apoptosis in TDECs

We have also previously examined the effects of 1,25-$(OH)_2D_3$ alone (26) and in combination with DEX (23, 25) on several key growth and proliferation signaling molecules in SCC tumor cells. Here, we tested whether treatment with 1,25-$(OH)_2D_3$ and/or DEX caused similar changes in the highly 1,25-$(OH)_2D_3$-sensitive $TDEC_{SCC}$ and whether these responses occurred in the relatively 1,25-$(OH)_2D_3$-insensitive MAE. As we reported for SCC cells (23), 1,25-$(OH)_2D_3$ caused a reduction in $p21^{Waf1}$ levels and an increase in $p27^{Kip1}$ in $TDEC_{SCC}$ (Fig. 4A). In contrast, 1,25-$(OH)_2D_3$ had no effect on $p21^{Waf1}$ and $p27^{Kip1}$ in MAE. The addition of DEX had little effect on $p21^{Waf1}$ and $p27^{Kip1}$ in $TDEC_{SCC}$, but did have a modest effect in MAE.

1,25-$(OH)_2D_3$ treatment also caused a decrease in the levels of phosphorylated, active ERK and Akt and led to the hypophosphorylation of Rb in $TDEC_{SCC}$ (Fig. 4B). These effects also occur in SCC cells and are consistent with a G_0/G_1 cell cycle arrest (25). These markers were unchanged in MAE cells treated with 1,25-$(OH)_2D_3$. Although DEX modestly enhanced the effects of 1,25-$(OH)_2D_3$ on phospho-ERK1/2 and had little effect on phospho-Akt and Rb in $TDEC_{SCC}$, DEX treatment resulted in a reduction of both phospho-Akt and the hyperphosphorylated form of Rb in MAE.

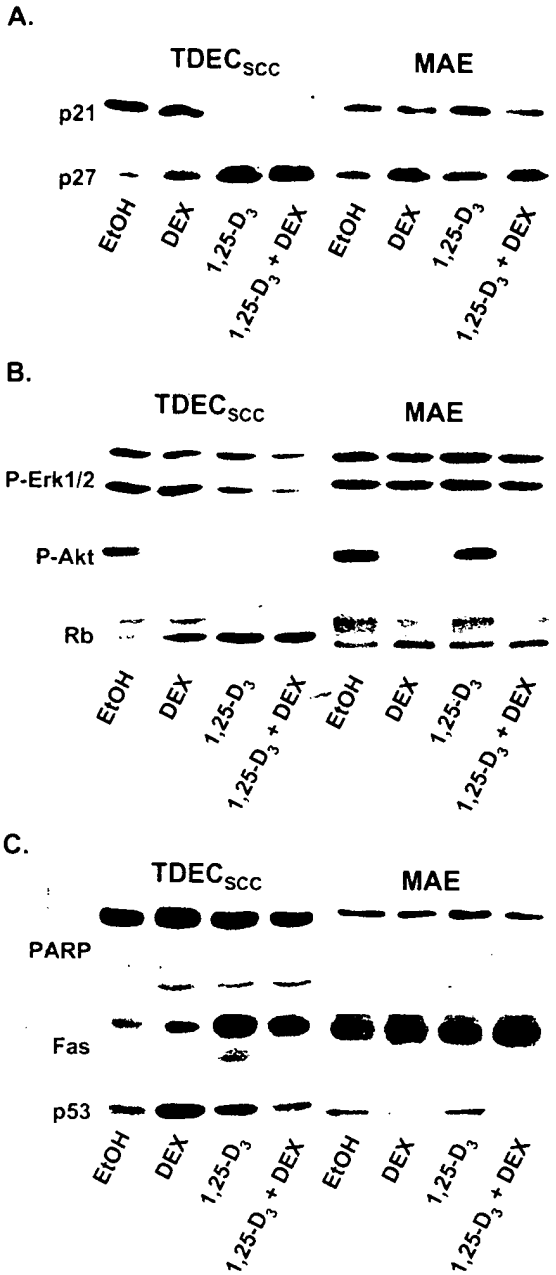

FIG. 4. Effects of 1,25-$(OH)_2D_3$ and DEX on markers of cell cycle, proliferation, survival, and apoptosis in $TDEC_{SCC}$ and MAE. $TDEC_{SCC}$ and MAE were treated with ethanol vehicle control, 1,25-$(OH)_2D_3$ (10 nM) with or without DEX (500 nM), or DEX alone for 48 h. Whole cell lysates were prepared, and equal amounts of protein were loaded for each treatment group. Western blots were then performed for the cyclin-dependent kinase inhibitors, p21 and p27 (A); P-ERK1/2, P-Akt, and Rb (B); and PARP [116-kDa full-length (*upper band*) and 85-kDa cleavage fragment (*lower band*)], Fas, and p53 (C). The data shown are representative of at least two independent experiments.

As 1,25-$(OH)_2D_3$ has been shown to induce apoptosis in some cell types (2, 27), including SCC cells (26), we examined whether induction of apoptosis contributes to the antiproliferative effects observed in $TDEC_{SCC}$. A small increase in the 85-kDa PARP cleavage fragment, indicative of apoptosis, was observed in $TDEC_{SCC}$ treated with either 1,25-$(OH)_2D_3$ or DEX compared with control, whereas the combination resulted in the same amount of PARP cleavage as the single agents (Fig. 4C). Evidence of apoptosis in $TDEC_{SCC}$ after treatment with 1,25-$(OH)_2D_3$ or the combination of 1,25-$(OH)_2D_3$ and DEX was also observed using an annexin V binding assay; however, across multiple experiments, the extent of apoptosis was variable, and no statistically significant difference between 1,25-$(OH)_2D_3$ alone and the combination was observed (data not shown). Further, no changes were observed in levels of the apoptotic regulators Bax, Bcl-2, and Fas ligand (data not shown). However, the expression of Fas and p53 was modulated. 1,25-$(OH)_2D_3$ treatment led to an increase in Fas levels in $TDEC_{SCC}$ with little effect in MAE (Fig. 4C). 1,25-$(OH)_2D_3$ or DEX alone also induced p53 levels in $TDEC_{SCC}$, whereas the effect of the combination was similar to the control level (Fig. 4C). In contrast, DEX alone or in combination with 1,25-$(OH)_2D_3$ caused a reduction in p53 in MAE, whereas 1,25-$(OH)_2D_3$ alone had little effect.

1,25-$(OH)_2D_3$ treatment decreases expression of Ang-2 in cancer cells

To test whether 1,25-$(OH)_2D_3$ and DEX affect angiogenesis by modulating the expression of angiogenic signaling molecules, a targeted cDNA array containing 25 murine, angiogenesis-related genes (Nonrad-GEArray, SuperArray, Inc., Bethesda, MD) was used to assess changes in expression after treatment with 1,25-$(OH)_2D_3$ and DEX. Although no significant differences were detected with 1,25-$(OH)_2D_3$ treatment in $TDEC_{SCC}$, Ang-2 levels were reduced in SCC cells after treatment with 1,25-$(OH)_2D_3$ and DEX (data not shown). This result was confirmed by Western blot analysis of SCC whole cell lysates, which demonstrated that the modulation of Ang-2 levels also occurs at the protein level (Fig. 5). The reduction was more dramatic after treatment with 1,25-$(OH)_2D_3$ alone than after the combination of 1,25-$(OH)_2D_3$ and DEX. Similar results were observed in RIF-1 cells (Fig. 5).

In contrast to the immunohistochemistry studies by Iseki *et al.* (11) demonstrating that treatment with 1,25-$(OH)_2D_3$ decreased the expression of VEGF in a colon cancer model, we did not detect a significant change in VEGF RNA levels in our array analysis (data not shown). However, VEGF was

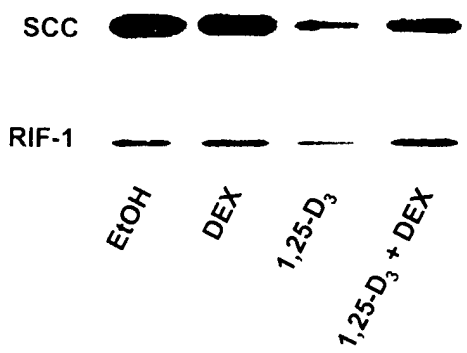

FIG. 5. 1,25-$(OH)_2D_3$ decreases angiopoietin-2 protein levels. Western blot analysis of SCC and RIF-1 whole cell lysates treated with ethanol vehicle control, 1,25-$(OH)_2D_3$ (10 nM) with or without DEX (500 nM), or DEX alone for 48 h using an antibody specific for murine Ang-2. The data shown are representative of two independent experiments.

only expressed at low levels in untreated SCC cells; therefore, this model may not be optimal for exploring the effects of VEGF modulation.

Discussion

Despite strong evidence that 1,25-$(OH)_2D_3$ has antitumor activity in a wide variety of tumor types, including prostate, colon, breast, and others (2, 28, 29), it is still unclear how to translate these results into a useful therapeutic regimen in cancer patients. 1,25-$(OH)_2D_3$ and vitamin D analogs are currently being evaluated in phase I and phase II clinical trials as both single agents and in combination with cytotoxic agents in a variety of cancer types. However, the most appropriate dose and schedule have not been defined, nor is it known which cancer types, let alone which individual patients, will respond to vitamin D-based therapies. Although clinical trials will certainly help to address these issues, information regarding the antitumor mechanism(s) of 1,25-$(OH)_2D_3$ and vitamin D analogs would also be helpful. For example, recent evidence that some cytotoxic agents, such as paclitaxel (30), vinca alkaloids (31), and camptothecins (32), also affect angiogenesis has led to new hypotheses about the most appropriate dosing strategies for these agents (33). Similar ideas should be considered for 1,25-$(OH)_2D_3$ if it also inhibits tumor angiogenesis.

In fact, there is considerable data to suggest that 1,25-$(OH)_2D_3$ and vitamin D analogs can inhibit angiogenesis (9–12). However, it is also important to understand whether the antiangiogenic effects occur in both tumors and normal tissues, and whether these effects occur by direct actions on endothelial cells, via indirect effects on angiogenic signaling, or a combination of both mechanisms. Endothelial cells and the unique signaling pathways that they use have become attractive targets for new anticancer agents; therefore, understanding the mechanism(s) by which 1,25-$(OH)_2D_3$ inhibits tumor angiogenesis would potentially allow a more rational basis for designing combination therapies involving such agents. Further, identification of the signaling pathways that are involved would potentially provide useful biomarkers of response to therapy, as has been proposed for other antiangiogenic agents (34).

Previous studies have demonstrated various effects of 1,25-$(OH)_2D_3$ on endothelial cell types derived from normal tissues (8, 12, 35–37). However, endothelial cells within tumors are not the same as those in normal tissues; therefore, it is critical to use models that are representative of this unique setting when examining the effects of an agent on tumor angiogenesis. Although umbilical vein and aortic endothelial cells are commonly used to test agents for antiangiogenic effects, we and others have identified selective markers for TDECs (15, 16), several of which are conserved in mice and humans (38). In addition to these molecular differences, there are clear morphological differences between tumor vessels and normal vessels, as tumor vessels are more tortuous, leaky, and often lack pericytes (39–41). Thus, we examined the effects of 1,25-$(OH)_2D_3$ on both TDECs and endothelial cells from normal tissues to determine whether there were differences in responsiveness to 1,25-$(OH)_2D_3$ among endothelial cells of different origins.

In fact, considerable variation was observed in responsiveness to the antiproliferative effects of 1,25-$(OH)_2D_3$ among these endothelial cell types. Interestingly, the two TDEC types were more sensitive than the endothelial cells from the normal tissues that were examined. Although the potency of 1,25-$(OH)_2D_3$ on both $TDEC_{SCC}$ and $TDEC_{RIF}$ was comparable to what was observed for the corresponding tumor cells (3), MAE and MYSEC were relatively resistant. Thus, there is a broad range of sensitivity among endothelial cells in different settings. Although only a few endothelial cell types have been examined to date, if a similar selectivity for endothelial cells in tumors compared with normal tissues exists *in vivo*, there may be a therapeutic window that would allow inhibition of tumor angiogenesis without adverse effects on normal vasculature. In addition, it is possible that 1,25-$(OH)_2D_3$ may have stronger effects in a local environment where endothelial cells are stimulated to proliferate, such as in tumors, than in situations where proliferation is only needed at a maintenance level. However, further studies are necessary to more precisely define which endothelial cell types are sensitive to 1,25-$(OH)_2D_3$.

Unfortunately, the use of 1,25-$(OH)_2D_3$ as a single agent therapy is limited by hypercalcemia and hypercalcuria. Thus, less hypercalcemic analogs or a combination of 1,25-$(OH)_2D_3$ with agents that can abrogate the hypercalcemia, such as DEX, hold more promise for clinical applications. Both of these strategies have demonstrated greater antiproliferative effects on tumor cells and antitumor activity *in vivo* than 1,25-$(OH)_2D_3$ alone (20, 42, 43). In addition, EB1089 and the combination of 1,25-$(OH)_2D_3$ and DEX are being evaluated in clinical trials. Here, we demonstrate that improved antiproliferative effects against TDECs can be achieved using the same drug concentration by either replacing 1,25-$(OH)_2D_3$ with a vitamin D analog or combining 1,25-$(OH)_2D_3$ with DEX.

Although the strong antiproliferative effects of the vitamin D analogs are promising for the clinical development of these compounds, these results may not provide much insight about the structural requirements for further analog optimization. For example, the antiproliferative activity of the analogs did not correlate with their VDR binding affinity, as all of the analogs have lower VDR affinity than the parent (with EB1089 being weakest) (44), but equivalent or stronger antiproliferative effects. On the other hand, our antiproliferative results roughly correlate with the ratio of VDR affinity to vitamin D-binding protein affinity (44), at least for 7553, 6760, and 1,25-$(OH)_2D_3$. The affinity of EB1089 for vitamin D-binding protein, and therefore the ratio, are not known. However, several other factors are potentially important, such as their relative ability to enter cells, their catabolic inactivation, as well as their ability to stabilize VDR in a conformation that heterodimerizes with RXR, binds DNA, promotes binding of coactivators, and ultimately regulates transcription (45). Several of these factors may also vary depending on the cell type or tissue being tested. Thus, although our results are not inconsistent with previous data, they could not have been easily predicted.

As we observed variations in the responsiveness to 1,25-$(OH)_2D_3$ among endothelial cell types derived from different tissues, we examined whether the sensitivity to 1,25-$(OH)_2D_3$

could be explained by differences in the expression of the VDR. As was the case in SCC cells (25), treatment of $TDEC_{SCC}$ with 1,25-$(OH)_2D_3$ caused an increase in VDR protein levels, an effect that was enhanced by the addition of DEX. This ability of DEX to further induce VDR levels may be important for the enhanced antitumor effects seen with this combination if it results in increased VDR signaling. In contrast, only low levels of VDR were observed in MAE after treatment with 1,25-$(OH)_2D_3$ alone or in combination with DEX compared with either $TDEC_{SCC}$ or $TDEC_{RIF}$. These results correlate with the responses of these cell types to 1,25-$(OH)_2D_3$ in the crystal violet assay. However, MYSEC expressed similar levels of VDR as TDEC after treatment with 1,25-$(OH)_2D_3$, but were significantly less sensitive in the crystal violet assay, suggesting that additional factors are involved. Nevertheless, the expression of VDR in endothelial cells lining the tumor vasculature may be a positive predictor of the response to 1,25-$(OH)_2D_3$.

We have also previously examined the effects of 1,25-$(OH)_2D_3$ alone (26) and in combination with DEX (23, 25) on certain key growth and proliferation signaling molecules in SCC tumor cells. Here, we tested whether treatment with 1,25-$(OH)_2D_3$ and/or DEX caused similar changes in the highly 1,25-$(OH)_2D_3$-sensitive $TDEC_{SCC}$ and whether these responses did not occur in the relatively 1,25-$(OH)_2D_3$-insensitive MAE. In general, similar effects were observed in the $TDEC_{SCC}$ as in the SCC cells, but not in the MAE. Thus, the expression of high levels of VDR may be necessary to respond to low concentrations of 1,25-$(OH)_2D_3$, as the MAE cells expressed only low levels of VDR.

Both cell cycle and apoptotic effects were observed and probably contribute to the antiproliferative activity. Effects such as inhibition of the ERK and Akt pathways are of interest because they may sensitize the tumor endothelium to other chemotherapy agents (46, 47) and serve as potential biomarkers of response. On the other hand, the induction of p53 by 1,25-$(OH)_2D_3$ in $TDEC_{SCC}$ differs from the decrease observed in SCC cells (Hershberger, P. A., T. F. McGuire, and C. S. Johnson, manuscript in preparation) and may be important in the rational design of combination therapies involving agents that act via p53-dependent mechanisms.

We also identified Ang-2 as a target molecule that is reduced in SCC and RIF-1 cells after treatment with 1,25-$(OH)_2D_3$. Ang-2 is an antagonist for the Tie-2 receptor (48); this ligand is overexpressed in a variety of tumors (49–51), and forced overexpression can promote tumor growth (52). Furthermore, Ang-2 can promote endothelial cell survival via the Akt pathway (53). Thus, the reduction in Ang-2 levels caused by 1,25-$(OH)_2D_3$ may inhibit tumor angiogenesis and overall tumor growth.

In conclusion, we have demonstrated that 1,25-$(OH)_2D_3$ alone or in combination with DEX can inhibit the proliferation of TDECs at low nanomolar concentrations, whereas two endothelial cell lines from normal tissues were significantly less sensitive. The expression of VDR could not completely explain these differences. In addition, three vitamin D analogs that have less propensity to induce hypercalcemia than 1,25-$(OH)_2D_3$ were at least as potent as the parent compound. Furthermore, the effects of 1,25-$(OH)_2D_3$ and DEX on signaling pathways, including ERK, Akt, and the cyclin-dependent kinase inhibitors, $p21^{Waf1}$ and $p27^{Kip1}$, were similar to our previous observations in SCC cells, whereas p53 was induced only in the TDECs. In contrast, 1,25-$(OH)_2D_3$ had minimal effects on these signaling molecules in MAE; therefore, there is considerable variation in the response of endothelial cells derived from different tissues. It will be interesting to note in future studies whether toxicities of 1,25-$(OH)_2D_3$ occur in tissues in which the endothelial cells are relatively sensitive. Finally, 1,25-$(OH)_2D_3$ also modulates the expression of Ang-2 in SCC and RIF-1 tumor cells, an effect that may inhibit tumor angiogenesis *in vivo*. Thus, our work supports the hypothesis that angiogenesis inhibition plays a role in the antitumor effects of vitamin D-based therapies, and that both direct effects on endothelial cells and indirect effects are involved. This information should be useful in the development of new combination therapies and in the design of future clinical trials.

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